Selective fermentation of gentiobiose-derived oligosaccharides by human gut bacteria and influence of molecular weight

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Abstract

Gentiooligosaccharides and alternansucrase gentiobiose acceptor products were fractionated by their degree of polymerization (DP) on a Bio-Gel P2 column. Fractions were characterized by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy, and incubated with human faecal bacteria under anaerobic conditions at 37 °C. The growth of predominant gut bacteria on the oligosaccharides was evaluated by fluorescence *in situ* hybridization and a prebiotic index (PI) was calculated. Lower DP gentiooligosaccharides (DP2–3) showed the highest selectivity (PI of 4.89 and 3.40, respectively), whereas DP4–5 alternansucrase gentiobiose acceptor products generated the greatest values (PI of 5.87). The production of short-chain fatty acids was also determined during the time course of the reactions. The mixture of DP6–10 alternansucrase gentiobiose acceptor products generated the highest levels of butyric acid but the lowest levels of lactic acid. Generally, for similar molecular weights, alternansucrase gentiobiose acceptor products gave higher PI values than gentiooligosaccharides.

Introduction

Prebiotics are dietary ingredients which should possess the three following criteria: (1) be resistant to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption; (2) be fermented by the intestinal microflora; and (3) stimulate selectively the growth and/or activity of intestinal bacteria associated with health and wellbeing (Gibson and Roberfroid, 1995; Gibson *et al.*, 2004). Some carbohydrates, such as fructooligosaccharides (FOS), inulin and galactooligosaccharides (GOS), are well-accepted prebiotics. However, there are still many oligosaccharides under investigation for their prebiotic potential. This is the case for the gentiooligosaccharides (GEOS) among others, which are β(1–6)-linked glucose polymers. Such compounds are not hydrolysed in the stomach or small intestine (Playne & Crittenden, 1996) and therefore reach the colon intact. In addition, their bitter taste makes them useful as taste-improvers for certain beverages (Côté *et al.*, 2003).

Recently, *in vitro* studies have demonstrated that GEOS have bifidogenic activity higher than that of FOS (Rycroft *et al.*, 2001). However, fermentation of GEOS is not as selective as that of FOS, and their prebiotic status therefore remains in doubt.

The metabolic end products from one microbial group can be used as a substrate for others, and some microorganisms benefit from substrates which they are not able to ferment directly (Gibson & Roberfroid, 1995). Therefore, to increase our knowledge of the fermentation selectivity of GEOS, it would be useful to determine the influence of different molecular weight fractions using mixed culture inocula.

Promising sources of prebiotic oligosaccharides are those obtained by enzymatic synthesis using alternansucrases (ASRs) (Holt *et al.*, 2005; Sanz *et al.*, 2005a). Such enzymes are extracellular glucansucrases isolated from *Leuconostoc mesenteroides* NRRL B-1355, which catalyses reactions between sucrose and low-molecular-weight acceptor carbohydrates (Côté & Robyt, 1982a, b). Oligosaccharides with both $\alpha(1-6)$- and $\alpha(1-3)$-linked acceptor products are synthesized from these reactions. The selective growth of purportedly beneficial bacteria using alternan maltose acceptor products has been reported recently (Sanz *et al.*, 2005a).

In this work, the effect of GEOS and alternan gentiobiose acceptor oligosaccharides on the selective growth of faecal bacteria was studied. In addition, the influence of molecular weight and linkage structure was investigated.

Materials and methods

Alternansucrase

Alternansucrase was isolated from sucrose-grown cultures of *Leuconostoc mesenteroides* NRRL B-21297. Cell-free culture fluid was concentrated by ultrafiltration using a 100 000 nominal molecular weight cut-off membrane and dialysed against 20 mM pH 5.4 sodium acetate buffer. The only glycansucrase activity detected in this concentrate was ASR (Côté *et al.*, 2003).

Carbohydrates

Sucrose was purchased from Sigma Co. (Poole, UK). FOS (Raftilose P-95) were acquired from Orafti (Tienen, Belgium) and GEOS from Wako Pure Chemicals (Osaka, Japan).

Acceptor reaction conditions

Acceptor reactions were carried out at room temperature in 20 mM, pH 5.4 sodium acetate buffer containing 0.01% (weight in volume, w/v) sodium azide, as described previously (Côté *et al.*, 2003). Reactions were terminated when all sucrose had been consumed, typically after 24–48 h.

Characterization of oligosaccharides

Oligosaccharides were separated using a Bio-Gel P2 (fine mesh) column (5×150 cm) (BioRad, Watford, UK), eluted with water under gravity flow. Each fraction was detected by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using a Bruker Daltonics Omniflex spectrometer (Bremen, Germany). Aqueous solutions of oligosaccharides were mixed with an equal volume of saturated 2,5-dihydroxybenzoic acid solution in acetonitrile, allowed to dry on the probe and subjected to MALDI-TOF mass spectrometry.

In vitro fermentations

In vitro fermentations were carried out as described previously (Sanz *et al.*, 2005b). Carbohydrates (7 mg) were dissolved in autoclaved nutrient basal medium to give a final concentration of 1% (w/v). Samples were then inoculated with 70 μL of slurry prepared by homogenizing fresh human faeces from healthy donors (10%, w/v) in phosphate-buffered saline (PBS; 8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.15 g L^{-1} Na_2HPO_4 and 0.2 g L^{-1} KH_2HPO_4, pH 7.3) (Oxoid, Basingstoke, UK) with a manual homogenizer (Fisher, Loughborough, UK) inside an anaerobic cabinet (10% H_2, 10% CO_2, 80% N_2). Three donors were used who did not have any history of gastrointestinal disorders and had avoided probiotics, prebiotics and antibiotics for at least 3 months prior to the study. Fermentations were carried out in triplicate at 37 °C. One sample was prepared without any carbohydrate addition as a control. All additions, inoculations and incubations were conducted inside an anaerobic cabinet (10% H_2, 10% CO_2, 80% N_2). Samples (200 μL) were removed after 0 and 12 h of fermentation for the enumeration of bacteria and short-chain fatty acid (SCFA) analysis.

Enumeration of bacteria

Bacteria were counted using fluorescence *in situ* hybridization (FISH). Samples (100 μL) were fixed overnight at 4 °C with 4% (w/v) filtered paraformaldehyde (pH 7.2) in a ratio of 1 : 3 (volume in volume, v/v). Samples were then washed twice with filtered PBS, resuspended in 200 μL of a mixture of PBS–ethanol (1 : 1, v/v) and stored at −20 °C until further analysis. Hybridization of the samples was carried out as described previously (Rycroft *et al.*, 2001) using appropriate genus-specific 16S rRNA-targeted oligonucleotide probes labelled with the fluorescent dye Cy3 (MWG Biotech, Ebersberg, Germany) for the different bacteria, or the nucleic acid stain 4′,6-diamidino-2-phenylindole (DAPI) for total cell counts. The probes used for each of the bacteria, previously validated by different authors, were Bif164, specific for *Bifidobacterium* (Langedijk *et al.*, 1995), Bac303, specific for *Bacteroides* (Manz *et al.*, 1996), His150, specific for *Clostridium* (*histolyticum* subgroup) (Franks *et al.*, 1998), EREC482, specific for *Eubacterium* (*Clostridium coccoides–Eubacterium rectale* group) (Franks *et al.*, 1998), Lab158, specific for *Lactobacillus/Enterococcus* (Harmsen *et al.*, 1999), and ATO291, specific for *Atopobium* (*Coriobacterium group*) (Harmsen *et al.*, 2000). The samples were then filtered using 0.2 μm pore size filters (Millipore Corporation, Watford, UK) and cells were counted using a Nikon Eclipse E400 fluorescence microscope (Nikon, Kingston upon Thames, UK). A minimum of 15 random fields was counted in each slide.

Analysis of SCFA and lactic acid

Samples were centrifuged at 13 000 ***g*** for 5 min and 20 μL was injected onto the high performance liquid chromatography (HPLC) system (Hewlett-Packard HP1050 series, Agilent, Wokingham, UK) equipped with an ultraviolet (UV) detector and an automatic injector. The column was an ion-exclusion Aminex HPX-87H (7.8×300 mm, BioRad) maintained at 50 °C. The eluent was 0.005 mM sulphuric acid in HPLC-grade water and the flow was 0.6 mL min^{-1}. Detection was performed at 210 nm and data were acquired using Chem Station for LC3D software (Agilent Technologies). Quantification of the samples was carried out using calibration curves for acetic, propionic, butyric and lactic acids at concentrations between 0.5 and 100 mM.

Prebiotic index (PI)

To obtain a general quantitative comparative measure of the selectivity of fermentation and to compare the influence of size and structure in the selective fermentation, a PI was calculated. The PI has previously been reported in the literature as a relationship between changes in the 'beneficial' and 'undesirable' elements within the microflora, all related to their starting levels (Olano-Martin *et al*., 2002; Palframan *et al*., 2003; Sanz *et al*., 2005c). The equation used was therefore as follows:

$$\begin{aligned} \mathrm{PI} &= \alpha + \beta + \gamma - \delta - \varepsilon \\ \alpha &= (Bif12/Bif0)/\mathrm{total} \\ \beta &= (Lac12/Lac0)/\mathrm{total} \\ \gamma &= (EREC12/EREC0)/\mathrm{total} \\ \delta &= (Bac12/Bac0)/\mathrm{total} \\ \varepsilon &= (His12/His0)/\mathrm{total} \end{aligned}$$

where total = total count (12 h)/total count (0 h), *Bif*12 = bifidobacterial count at 12 h, *Bif*0 = bifidobacterial count at 0 h, etc. No changes were observed in *Atopobium* spp. and therefore it was not included in this equation.

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 12.0.1 (SPSS Inc., Chicago, IL). Univariate analysis of variance (ANOVA) and least significant difference (LSD) test were also used to determine significant differences between the bacterial populations using the different oligosaccharides. The differences were considered to be significant when $P < 0.05$.

Results

Oligosaccharide characterization

The composition of the oligosaccharide fractions separated by the Bio-Gel P2 column was identified by MALDI-TOF mass spectrometric analysis (Fig. 1). For GEOS (Fig. 1a), fraction 1 was composed solely of gentiobiose (GEOS1). GEOS2 was mainly composed of the degree of polymerization 3 (DP3) fraction, although DP2, DP4 and DP5 were also present. GEOS3 and GEOS4 were composed of DP4, DP5, DP6, DP7 and DP8, with the main constituents being DP4 and DP5, respectively. GEOS5 was also composed of DP5–DP10, with the main constituents being DP6 and DP7.

For the alternansucrase gentiobiose acceptor products (AGOS) (Fig. 1b), fraction 1 (AGOS A) was composed mainly of DP3, with some DP4 also present. AGOS B and AGOS C were formed mainly by DP4 and DP6, respectively. AGOS D was composed of DP6–DP11, with the main constituents being DP6, DP7 and DP8. Previous results (Holt *et al*., 2005) have indicated that only a single DP3 product is formed, which was identified as α-D-glucopyranosyl-(1–6)-β-D-glucopyranosyl-(1–6)-D-glucopyranose. Two oligosaccharides of DP4 were obtained and identified as $[\alpha$-D-glucopyranosyl-(1–6)$]_2$-β-D-glucopyranosyl-(1–6)-D-glucopyranose and α-D-glucopyranosyl-(1–3)-α-D-glucopyranosyl-(1–6)-β-D-glucopyranosyl-(1–6)-D-glucopyranose. Higher DP products have not yet been structurally characterized.

Batch culture fermentations

Table 1 shows the changes in the selected bacterial populations during the incubation of the different fractions of oligosaccharides with a faecal inoculum. FOS and a commercial mixture of GEOS were also included in this study. All the samples used showed a significant increase in bifidobacterial populations, with AGOS C presenting the greatest value. A significant increase in bacteroides was detected in GEOS5, whereas AGOS D and GEOS resulted in the highest populations of clostridia. The *Lactobacillus* population did not vary significantly from the initial sample; however, some changes could be seen in the samples. FOS and GEOS showed the greatest populations, whereas GEOS5 presented the lowest. *Atopobium* spp. did not show significant differences during the fermentation process.

A PI was calculated for each sample to obtain a quantitative measure of the degree of selectivity of fermentation. Figure 2 shows the values obtained for these PIs. GEOS2 and AGOS B revealed the highest PI values, even greater than FOS. The higher molecular weight oligosaccharides GEOS4 and GEOS5 presented the lowest PIs due to increases in clostridia and low values of lactobacilli.

Table 2 shows the SCFA and lactic acid compositions. Lactic and acetic acids, which are mainly produced by bifidobacteria, showed the lowest values for GEOS5, AGOS C and AGOS D. The latter sample showed the largest amount of propionic and butyric acids, followed by GEOS5 and GEOS1.

Discussion

Some authors have indicated the potential prebiotic effect of GEOS (Rycroft *et al*., 2001); however, more *in vitro* and *in vivo* studies are necessary to confirm their functional properties. As indicated in previous studies, GEOS showed a promoting effect on the growth of bifidobacteria (8.64 log). Although no significant variations were found for the growth of these bacteria amongst the different molecular weights, GEOS3, GEOS4 and GEOS5 showed the greatest values; however, these fractions also resulted in the highest clostridia and bacteroides populations. Previous studies (Rycroft *et al*., 2001) have shown that GEOS is not as selective as FOS, and that clostridia increase during *in vitro* fermentations. These results were confirmed in this study

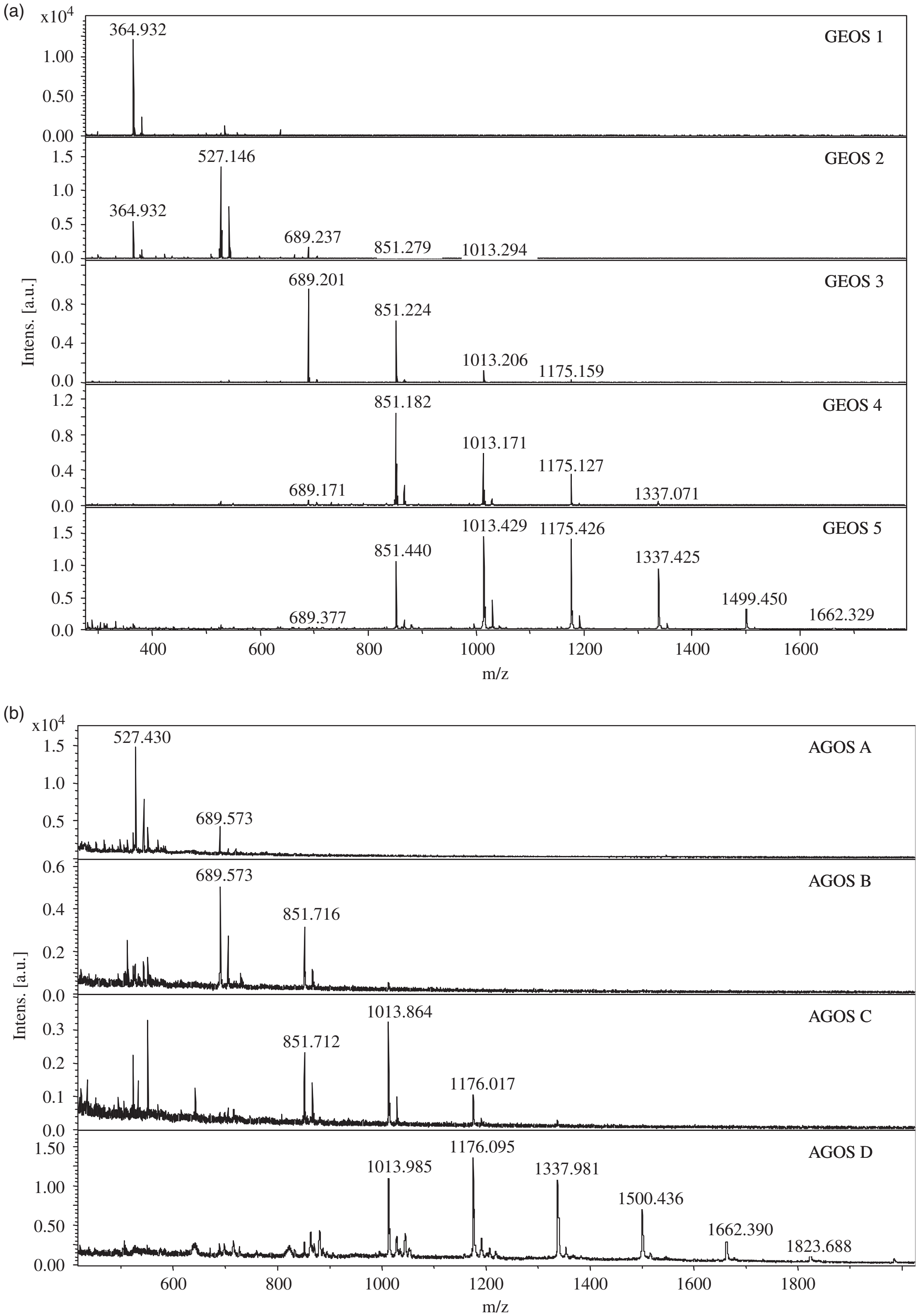

Fig. 1. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometric analysis of oligosaccharide fractions obtained after separation by Bio-Gel P2. (a) Gentiooligosaccharides (GEOS). (b) Alternansucrase gentiobiose acceptor products (AGOS).

Table 1. Changes in bacterial population (log cell mL^{-1}) after 12 h of *in vitro* fermentation incubation with fructooligosaccharides (FOS), gentiooligosaccharides (GEOS) and alternansucrase gentiobiose acceptor products (AGOS) ($n = 3$)

	Total bacteria	Bifidobacteria	Bacteroides	Clostridia	Eubacteria	*Lactobacillus*	*Atopobium*
0 h	9.2 (0.1)^{a}	7.9 (0.2)^{a}	8.0 (0.1)^{a}	6.9 (0.1)^{a,b}	8.2 (0.0)^{a,b}	6.9 (0.2)^{a,b,c}	7.5 (0.2)^{a}
Control	9.2 (0.0)^{a}	7.9 (0.1)^{a}	8.0 (0.1)^{a}	7.0 (0.0)^{a,b}	8.0 (0.1)^{a}	6.8 (0.1)^{a,b,c}	7.6 (0.1)^{a}
FOS	9.2 (0.1)^{a}	8.5 (0.1)^{b,c}	8.1 (0.0)^{a}	6.9 (0.2)^{a,b}	8.2 (0.1)^{a,b}	7.3 (0.2)^{a}	7.7 (0.2)^{a}
GEOS	9.2 (0.0)^{a}	8.6 (0.1)^{b,c,d}	8.1 (0.1)^{a}	7.5 (0.1)^{c}	8.1 (0.1)^{a}	7.1 (0.1)^{a,b,c}	7.7 (0.1)^{a}
GEOS1	9.1 (0.0)^{a}	8.4 (0.2)^{b,c}	8.2 (0.1)^{a}	6.8 (0.1)^{a}	8.4 (0.1)^{a,b}	7.0 (0.3)^{a,b,c}	7.7 (0.2)^{a}
GEOS2	9.1 (0.1)^{a}	8.4 (0.1)^{b,c}	8.1 (0.1)^{a}	7.1 (0.1)^{a,b,c}	8.4 (0.1)^{a,b}	7.3 (0.2)^{a}	7.6 (0.1)^{a}
GEOS3	9.5 (0.0)^{c}	8.6 (0.1)^{b,c,d}	8.2 (0.0)^{a}	7.2 (0.2)^{b,c}	8.3 (0.1)^{a,b}	7.2 (0.2)^{a,b}	7.7 (0.1)^{a}
GEOS4	9.4 (0.0)^{b,c}	8.5 (0.1)^{b,c}	8.1 (0.0)^{a}	7.2 (0.2)^{a,b,c}	8.0 (0.1)^{a}	6.7 (0.0)^{b,c}	7.4 (0.1)^{a}
GEOS5	9.5 (0.1)^{c}	8.7 (0.1)^{c,d}	8.4 (0.0)^{b}	7.2 (0.2)^{b,c}	8.3 (0.2)^{a,b}	6.6 (0.1)^{c}	7.8 (0.2)^{a}
AGOS A	9.3 (0.0)^{a,b}	8.5 (0.1)^{b,c}	8.2 (0.1)^{a}	7.3 (0.1)^{b,c}	8.2 (0.1)^{a,b}	7.1 (0.3)^{a,b,c}	7.5 (0.1)^{a}
AGOS B	9.2 (0.0)^{a}	8.7 (0.1)^{c,d}	8.1 (0.1)^{a}	7.0 (0.3)^{a,b}	8.1 (0.1)^{a}	7.2 (0.2)^{a,b}	7.4 (0.2)^{a}
AGOS C	9.5 (0.1)^{c}	8.9 (0.1)^{d}	8.0 (0.1)^{a}	7.0 (0.1)^{a,b}	8.3 (0.1)^{a,b}	6.7 (0.2)^{b,c}	7.6 (0.1)^{a}
AGOS D	9.2(0.1)^{a}	8.3 (0.1)^{b}	8.6 (0.1)^{b}	7.0 (0.1)^{a,b}	8.5 (0.1)^{b}	7.0 (0.3)^{a,b,c}	7.6 (0.1)^{a}

A control sample without carbohydrate source is also included. Different superscript letters indicate significant differences ($P < 0.05$) for each bacterial genus. Standard error in parentheses.

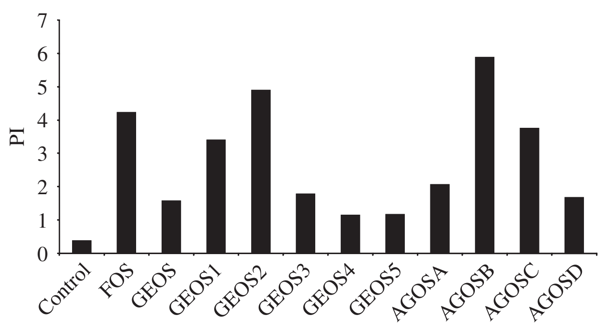

Fig. 2. Prebiotic index (PI) scores from batch cultures at 12 h and 37 °C using fructooligosaccharides (FOS), gentiooligosaccharides (GEOS) and alternansucrase gentiobiose acceptor products (AGOS) ($n = 3$).

for the mixture of GEOS (7.52 log); however, although a slight increase in clostridia was detected for most of the samples, when the different molecular weight fractions were considered independently, none showed a significant increase in these bacteria. Therefore, the prebiotic selectivity of GEOS was highest when they were separated into their different DPs, with GEOS1 and GEOS2 showing the greatest values.

Recently, studies using pure cultures of the AGOS mixture have demonstrated that these carbohydrates are selectively utilized by many species of *Bifidobacterium*, but do not support the growth of *Lactobacillus* spp., *Bacteroides thetaiotamicron*, coliforms or pathogenic bacteria (Holt *et al*., 2005). However, mixed culture work is essential to determine their prebiotic selectivity, as some bacteria can metabolize the end products of others (Gibson & Roberfroid, 1995). Moreover, previous data have not considered the influence of the molecular weight on the prebiotic effect. Our samples did not result in an increase in *Lactobacillus* populations; however, all promoted the growth of bifidobacteria, AGOS C generating the greatest value. However, the highest PI was found with AGOS B (DP4–5). AGOS D presented the greatest production of butyric acid and also the greatest increase in clostridia (8.57 log) and eubacteria (8.51 log), which are precursors of this SCFA.

Table 2. Short-chain fatty acid and lactic acid concentrations (mM) produced after 12 h of *in vitro* fermentation with fructooligosaccharides (FOS), gentiooligosaccharides (GEOS) and alternansucrase gentiobiose acceptor products (AGOS) ($n = 3$)

	Lactic	Acetic	Propionic	Butyric
0 h	2.2 (0.2)^{a}	0.4 (0.0)^{a}	0.1 (0.0)^{a}	0.1 (0.0)^{a}
Control	0.4 (0.0)^{a}	11.5 (0.8)^{a}	2.2 (0.3)^{b,c}	2.2 (0.2)^{b,c}
FOS	29.1 (2.1)^{d}	36.6 (2.6)^{b,c}	2.2 (0.2)^{b,c}	1.7 (0.5)^{a,b}
GEOS	38.6 (3.9)^{c}	45.8 (2.7)^{c,d}	2.4 (0.3)^{b,c}	1.9 (0.7)^{a,b,c}
GEOS1	27.1 (7.2)^{d}	33.4 (10.4)^{b}	3.3 (0.4)^{d,e}	3.6 (1.2)^{c,d}
GEOS2	34.1 (2.8)^{c,d}	45.9 (1.7)^{c,d}	2.0 (0.2)^{b}	2.1 (0.7)^{b,c}
GEOS3	30.8 (1.5)^{c,d}	50.4 (3.1)^{d}	2.5 (0.3)^{b,c}	2.5 (0.8)^{b,c}
GEOS4	27.0 (0.5)^{d}	49.6 (2.8)^{d}	2.6 (0.2)^{b,c}	2.6 (0.8)^{b,c}
GEOS5	13.2 (1.5)^{b}	36.7 (3.0)^{b,c}	3.8 (0.3)^{e}	3.4 (0.8)^{b,c}
AGOS A	34.6 (3.9)^{c,d}	49.9 (3.0)^{d}	2.3 (0.2)^{b,c}	1.8 (0.6)^{a,b,d}
AGOS B	34.0 (1.7)^{c,d}	53.1 (4.0)^{d}	2.4 (0.2)^{b,c}	1.8 (0.5)^{a,b,d}
AGOS C	8.4 (0.7)^{a,b}	45.4 (4.6)^{c,d}	2.8 (0.2)^{c,d}	2.4 (0.4)^{b,c}
AGOS D	0.4 (0.0)^{a}	26.8 (2.4)^{b}	7.1 (0.2)^{f}	3.8 (0.4)^{c}

A control sample without carbohydrate source is also included. Different superscript letters indicate significant differences ($P < 0.05$) for each acid. Standard error in parentheses.

In general, AGOS fractions with a similar molecular weight to GEOS showed higher prebiotic selectivity, apart from DP3. The presence of α linkages contributes towards the increased prebiotic activity. This effect has been seen in previous studies (Sanz *et al*., 2005b), in which glucobioses with α linkages had higher PI values than those with β linkages.

These studies have some value in predicting the potential prebiotic effect of gentiobiose-derived oligosaccharides.

Increased knowledge of the influence of the molecular weight on prebiotic selectivity may help to develop new products. On the basis of these data, GEOS and AGOS seem to be promising sources of prebiotic carbohydrates.

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